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Glyceraldehyde-3-Phosphate Dehydrogenase Is a Surface-Associated, Fibronectin-Binding Protein of *Trichomonas vaginalis*▽

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Trichomonas vaginalis **colonizes the urogenital tract of humans and causes trichomonosis, the most prevalent nonviral sexually transmitted disease. We have shown an association of *T. vaginalis* with basement membrane extracellular matrix components, a property which we hypothesize is important for colonization and persistence. In this study, we identify a fibronectin (FN)-binding protein of *T. vaginalis*. A monoclonal antibody (MAb) from a library of hybridomas that inhibited the binding of *T. vaginalis* organisms to immobilized FN was identified. The MAb (called ws1) recognized a 39-kDa protein and was used to screen a cDNA expression library of *T. vaginalis*. A 1,086-bp reactive cDNA clone that encoded a protein of 362 amino acids with identity to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained. The *gapdh* gene was cloned, and recombinant GAPDH (rGAPDH) was expressed in *Escherichia coli* cells. Natural GAPDH and rGAPDH bound to immobilized FN and to plasminogen and collagen but not to laminin. MAb ws1 inhibited binding to FN. GAPDH was detected on the surface of trichomonads and was upregulated in synthesis and surface expression by iron. Higher levels of binding to FN were seen for organisms grown in iron-replete medium than for organisms grown in iron-depleted medium. In addition, decreased synthesis of GAPDH by antisense transfection of *T. vaginalis* gave lower levels of organisms bound to FN and had no adverse effect on growth kinetics. Finally, GAPDH did not associate with immortalized vaginal epithelial cells (VECs), and neither GAPDH nor MAb ws1 inhibited the adherence of trichomonads to VECs. These results indicate that GAPDH is a surface-associated protein of *T. vaginalis* with alternative functions.**

Trichomonas vaginalis, an extracellular protozoan parasite, is the cause of trichomonosis, the most prevalent nonviral sexually transmitted disease (47). In women, vaginitis due to *T. vaginalis* clinically manifests with symptoms of vaginal itching, odor, and discharge. Adverse health outcomes for women with this sexually transmitted disease include cervical cancer (46) and preterm delivery and low-birth-weight infants (25). There is a relationship between seropositivity to *T. vaginalis* and prostate cancer (43). This disease is significant due to its association with human immunodeficiency virus (33, 45). More recently, persistent, undetected *T. vaginalis* infections associated with asymptomatic carriage were found among women (40).

T. vaginalis penetration of the mucous layer (28), followed by adherence to vaginal epithelial cells (VECs), is preparatory for colonization (9, 10). VEC adherence by parasites is mediated by numerous distinct trichomonad surface adhesins (5, 10, 18). Brief contact of *T. vaginalis* with VECs and fibronectin (FN) elicited dramatic changes in parasite morphology, suggesting a host-specific signaling of parasites (8, 9). Importantly, iron and cell contact by parasites each upregulated the expression of adhesins in a coordinated fashion via distinct mechanisms (2, 4, 6, 21, 29). Genetic approaches using antisense (AS) inhibition of synthesis (36, 37) and heterologous expression in *Tritrichomonas foetus* (26, 36) have reaffirmed the role of these *T. vaginalis* proteins as adhesins. *T. vaginalis* organisms secrete or release numerous metabolic enzymes, including adhesin AP65 (decarboxylating malic enzyme), α-enolase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) during growth and multiplication (27). AP65 and α-enolase were found to reassociate with the parasite surface for the expression of adhesin function (19) and binding to plasminogen (35), respectively.

There is an increased awareness of the existence of metabolic enzymes on the surfaces of bacterial pathogens, yeast, and parasites (12, 24, 35). These surface-associated enzymes appear to be novel virulence factors (17, 22, 38, 39). The anchorless glycolytic enzymes GAPDH (13, 31, 38) and α-enolase (39) are present on the surface of group A streptococcus. The surface-associated GAPDH of *Candida albicans* binds with strong affinity to FN and laminin (22). In enterohemorrhagic *Escherichia coli* and enteropathogenic *E. coli*, GAPDH is an extracellular protein that binds human plasminogen and fibrinogen and also interacts with intestinal epithelial cells (17).

We demonstrate that GAPDH is another enzyme on the surface of *T. vaginalis*. A monoclonal antibody (MAb) that inhibited parasite associations with FN was immunoreactive with GAPDH. Importantly, iron was found to regulate gene expression and synthesis and surface placement of GAPDH. Both low-iron-grown trichomonads and AS-transfected parasites with decreased amounts of GAPDH had smaller amounts of surface GAPDH and corresponding lower levels of binding to FN. GAPDH was not involved in adherence of trichomonads to immortalized VECs. Interestingly, as with other microbial pathogens, *T. vaginalis* GAPDH also bound plasminogen and collagen but not laminin (17, 22).

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MATERIALS AND METHODS

Parasite culture. *T. vaginalis* isolate UT0040 was grown in trypticase-yeast extract-maltose (TYM) medium with 10% heat-inactivated horse serum at 37°C. Trichomonads at the late logarithmic phase of growth (18 to 20 h) were labeled with calcein using the Vybrant cell adhesion assay kit (Invitrogen, Carlsbad, CA) for 30 min (20). For iron-replete parasites, TYM-serum was supplemented with 250 μM ferrous ammonium sulfate (Sigma-Aldrich Co., St. Louis, MO), and iron-depleted organisms were grown in medium with 50 μM 2,2-dipyridyl (Sigma) (15, 29) added to the batch culture medium. Parasite densities were determined by counting using a hemocytometer.

FN binding by trichomonads and adherence assays. Unless indicated otherwise, all assays were performed at least four times using triplicate samples. Ninety-six-well microtiter plates (Nunc) were coated with 1 μg FN (Gibco, Invitrogen) diluted in carbonate buffer (pH 9.6) and incubated overnight at 4°C. The wells were blocked by the addition of phosphate-buffered saline (PBS)–0.1% Tween 20 (PBS-T) containing 5% skim milk for 1 h at 37°C, followed by three washes with PBS-T. Parasites at the logarithmic phase of growth were washed in PBS and suspended to 2.5×10^6 parasites ml^{-1} in TYM for labeling, as described previously (19, 20), with calcein reagent (2 μl ml^{-1}) by using the Vybrant cell adhesion kit (Molecular Probes, Eugene, OR) for no less than 30 min according to the manufacturer's protocol. After washing the cells with PBS, 100 μl containing increasing numbers of trichomonads in the same volume as shown in Fig. 1A was added to the FN-coated wells and incubated for 30 min at 37°C. For standard binding assays, 5×10^4 cells were added to each well, as described previously (14). Wells with bound parasites were washed twice with cold minimal binding buffer (120 mM NaCl, 1.3 mM KCl, 0.9 mM NaH_2PO_4, 5.5 mM glucose, and 26 mM $NaHCO_3$ [pH 5.0]) (14), and the extent of binding was measured by fluorescence with a Synergy HT fluorometer (BioTek, Winooski, VT). This method permitted the optimization of parasite binding to microtiter wells.

For an adherence assay, trichomonads were labeled with calcein as described above. In a standard adherence assay, 4×10^5 organisms were added to confluent monolayers of immortalized MS74 VECs grown on Costar Stripwell 96-well microtiter plates (Corning, Inc., Corning, NY), as described previously (19–21). After 30 min of incubation at 37°C, wells were washed with warm PBS. One hundred microliters of PBS was then added to wells, and the extent of parasite binding was determined by fluorescence. Where indicated in antibody experiments, parasites were preincubated for 30 min with rabbit anti-AP65 serum, because AP65 has been found to be a prominent adhesin of *T. vaginalis* (20, 21). Pretreatment with normal rabbit serum served as a negative control. In other experiments, trichomonads were pretreated with 1 μg of purified recombinant GAPDH (rGAPDH) added to the suspension, or parasites were suspended in hybridoma supernatant diluted 1:2 (vol/vol) in TYM medium containing MAb ws1. The suspensions were then added to VEC monolayers, and numbers of adherent organisms were determined by fluorescence.

A library of MAbs against intact trichomonads was generated, as described previously (3), and hybridomas were screened for the inhibition of binding of *T. vaginalis* organisms to FN on microtiter plates. These hybridoma cells producing immunoglobulin G (IgG) were then single-cell cloned, and either hybridoma supernatant or purified IgG MAb was used for subsequent experiments. One IgG1 MAb (called ws1) inhibited parasite binding to FN. For MAb ws1 inhibition experiments, 5×10^4 trichomonads were pretreated with different dilutions of hybridoma supernatant containing MAb ws1 prior to addition to the FN-coated wells and were processed as described previously (15). A hybridoma supernatant with MAb L64, which is reactive with a trichomonad cytoplasmic protein (26), was used as a control.

cDNA library screening. A *T. vaginalis* cDNA expression library was constructed using the λ Zap II vector (35). The library was screened with MAb ws1. After two rounds of screening and plaque purification, phagemids were excised with Exassist interference-resistant helper phage according to the manufacturer's instructions. Sequencing was performed at the Molecular Biology Core Facility of the institution. The nucleotide sequence of the cDNA clone was translated into the corresponding amino acid sequence with the BioEdit program, followed by analysis using the BLAST program. Sequences were aligned using the Clustal W program.

Immunoblot analysis. Total proteins of 1×10^7 trichomonads were obtained by trichloroacetic acid precipitation (1) for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide. Proteins were electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) using a Transblot SD semidry transfer cell (Bio-Rad) and probed with MAb ws1 and MAb 12G4 against AP65 (21). The blots were further incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody, followed by washing and incubating with HRP substrate (Bio-Rad).

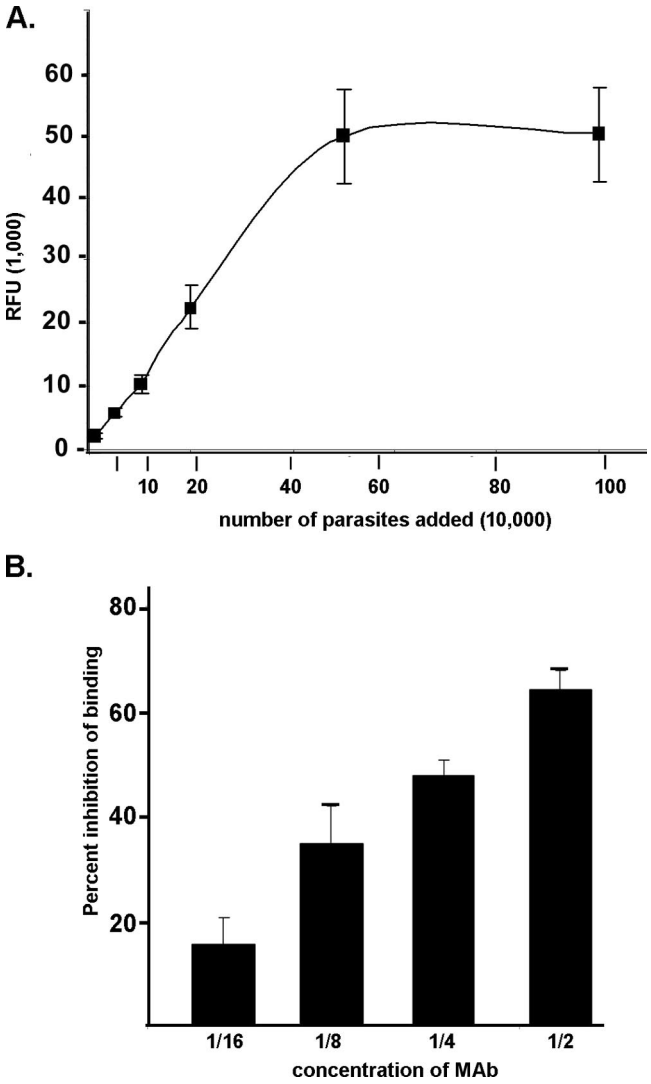

FIG. 1. Binding of *T. vaginalis* to FN and inhibition of binding by MAb ws1. (A) Representative experiment demonstrating kinetics of binding of *T. vaginalis* to FN immobilized in microtiter wells, as described in Materials and Methods. The parasites were labeled with calcein reagent, added to the FN-coated wells, and incubated for 30 min. The cells were washed, followed by measurement of fluorescence using a Synergy HT fluorometer. RFU, relative fluorescence units. (B) Inhibition experiment with parasites first pretreated with different concentrations of MAb ws1 determined by dilutions of hybridoma supernatant prior to the addition of the mixture to the FN-coated wells. Numbers of trichomonads bound were determined by measuring fluorescence as described above (A).

Expression and purification of His_6-rGAPDH. To express His_6::GAPDH in *E. coli*, the entire 1,086-bp *gapdh* open reading frame was PCR amplified from the cDNA clone by using *Taq* DNA polymerase (Invitrogen). The primers used were as follows: forward primer 5′-CTA CAG GTA CCG TAG TTA AAG TTG CTA TC-3′ and reverse primer 5′-GCA AGA AGC TTT AAA GAA TAC TTC TCA AG-3′ (restriction enzyme sites are underlined). The PCR conditions used were as follows: 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 48°C for 1 min, and 72°C for 1 min with a final extension step at 72°C for 10 min. The PCR product was digested with the restriction enzymes KpnI and HindIII and inserted into the KpnI-HindIII-digested and dephosphorylated expression vector pQE2 (TAGzyme vectors from Qiagen), which resulted in the recombinant plasmid pQE2-*gapdh*, expressing His_6::GAPDH. The identity of the construct was confirmed by DNA sequencing. pQE2-*gapdh* was transformed into M15 competent

bacterial cells by the heat shock method, and the transformants were named M15GAPDH cells. For purification of rGAPDH, M15GAPDH bacterial cells were then grown at 37°C in Luria-Bertani broth containing 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin to an optical density at 600 nm of 0.6, and expression was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C and at 225 rpm. Cells were harvested by centrifugation at 6,000 × *g* at 4°C, resuspended in STE buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], and 1 mM EDTA), and then sonicated on ice at full power using a Sartorius Labsonic M apparatus a total of two times at 45-s intervals. After centrifugation of the bacterial lysate twice for 30 min at 15,000 × *g*, the supernatant was discarded, and the pellet was solubilized in chromatography binding buffer (8 M urea, 500 mM NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole, and 50 mM Tris-HCl [pH 8.0]). The lysate was clarified by centrifugation before application onto a Ni-nitrilotriacetic acid column (Qiagen, Valencia, CA). After washing with binding buffer containing a decreasing linear gradient of urea, the column was subjected to elution buffer (500 mM NaCl, 5 mM β-mercaptoethanol, and 500 mM imidazole [pH 8.0]). The eluted His_6::GAPDH protein was then dialyzed against 1 liter of PBS for 12 to 16 h, and the PBS was changed every 4 h.

FN binding by rGAPDH. An enzyme-linked immunosorbent assay was performed to determine the ability of rGAPDH to bind wells of microtiter plates coated with 1 μg of FN. For controls, wells were coated with 5% skim milk or with 1 μg laminin. The plates were incubated overnight at 4°C. The wells were blocked with PBS-T containing 5% skim milk, followed by three washes with PBS-T. Different concentrations (0 to 400 ng) of rGAPDH diluted in PBS-T were added to the FN-coated wells and incubated for 1 h at 37°C. After washing twice with PBS-T, wells were then incubated with MAb ws1 followed by HRP-conjugated anti-mouse IgG for 1 h. Color was developed using HRP substrate. Absorbance readings were measured at 405 nm using a microplate reader (Bio Tek Instruments, Inc.). Similar binding assays were performed with 1 μg each of laminin, plasminogen, and collagen. A reverse binding assay was performed with 400 ng rGAPDH coated onto wells, followed by the addition of increasing concentrations of FN ranging from 1 μg to 4 μg each. Laminin was used in this reverse assay as a negative control. Binding was then determined by incubation with murine anti-FN antibody and HRP-conjugated anti-mouse secondary antibody. For antibody inhibition experiments, rGAPDH was pretreated for 30 min and incubated with different dilutions of hybridoma supernatant containing MAb ws1 prior to the addition of the mixture to the FN-coated plates.

Surface immunofluorescence detection of GAPDH. Immunofluorescence of nonpermeabilized trichomonads was carried out with a modification of a previously described procedure (21). Briefly, 1 × 10^6 trichomonads at the mid-to-late logarithmic phase of growth phase were washed twice with Ringer's solution and fixed with 2% paraformaldehyde for 10 min at room temperature. Trichomonads were blocked with 1% bovine serum albumin for 1 h at room temperature prior to incubation for 1 h with hybridoma supernatants and with MAb ws1 diluted 1:100. As a negative control, MAb L64 against a cytoplasmic protein (26) was used. Parasites were washed with Ringer's solution and incubated for 1 h at 37°C with affinity-purified fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma-Aldrich Co.) diluted 1:1,000. Finally, parasites were washed twice with Ringer's solution and observed at a magnification of ×100 using an Olympus BX41 microscope.

Generation of S and AS plasmids with the *gapdh* coding region. The sense (S) and AS plasmids, designated *pBS-neo-gapdh-S* and *pBS-neo-gapdh-AS*, respectively, were constructed by cloning the coding region of *gapdh* in forward (S primer 5′-CCGTATCATATGGTTGTCCTCGAGTCAACAGG-3′ and AS primer 5′-CTCATGGGTACCAAGGTACTTCTCAAGGCGGT-3′) and reverse (S primer 5′-CCGTATCATATGAAGGTACTTCTCAAGGCGGT-3′ and AS primer 5′-CTGATCGGTACCGGTGTCCTCGAGTCAACAGG-3′) orientations. The original plasmid, *pBS-FdHAHA-neo*, was used previously (37). S and AS plasmids were confirmed by sequencing and by PCR of known sequences.

RNA isolation and reverse transcription (RT)-PCR analysis. Total RNA was isolated from approximately 4 × 10^7 *T. vaginalis* cells using Trizol reagent (Invitrogen), as described previously (37). RNA was ethanol precipitated and reverse transcribed using SuperScript reverse transcriptase II and oligo(dT) primers (Invitrogen). cDNA was used as a template for the PCRs according to the manufacturer's protocol. The primers used for the PCR amplifications of the *gapdh* transcript were as follows: *gapdh* S primer 5′-GATCGGTACCATCCCA ACATCCACAGGTGCTGCTA-3′ and *gapdh* AS primer 5′-GCAAGAAGCTT TTAAAGATACTTCTCAAG-3′. The α-tubulin gene S primer was 5′-ACTCT GCTGCCTCGAGCACGGTATC-3′, and the AS primer was 5′-GAAATGAC TGGTGCATAAAGAGC-3′.

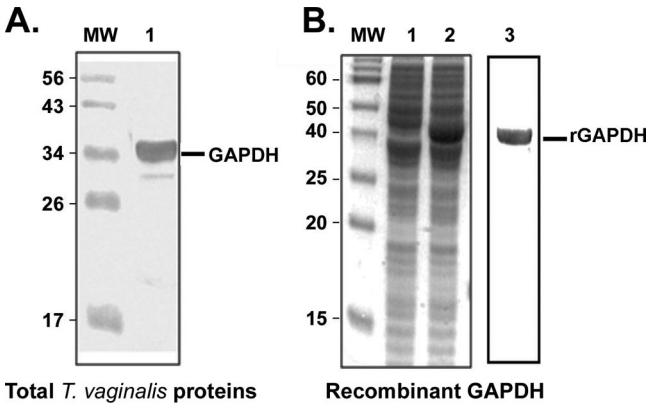

FIG. 2. Detection of a 39-kDa band in the total cell lysate of *T. vaginalis*. (A) The total proteins of *T. vaginalis* were trichloroacetic acid precipitated and electrophoresed on 10% SDS-PAGE gels, followed by blotting of proteins onto nitrocellulose membranes for probing with MAb ws1. Lane 1 shows the intensity of the GAPDH protein band detected by MAb ws1. (B) Cloning of *gapdh* cDNA into vector pQE2 for expression as His_6-rGAPDH in *E. coli.* Lane 2 shows the stained total protein pattern of *E. coli* cells expressing GAPDH compared with total proteins of *E. coli* with vector without insert (lane 1). Lane 3 presents the immunoblot of a duplicate of lane 2 probed with MAb ws1. The larger His_6 fusion GAPDH protein was expected. MW refers to the stained molecular weight standards (in thousands) electrophoresed identically during the experiment.

Transfection and selection for G418 resistance. Transfection of *T. vaginalis* parasites at the early logarithmic phase of growth was carried out by electroporation, as described previously (36, 37, 44). Briefly, 4 × 10^7 parasites were centrifuged at 1,800 rpm at 4°C, and the pellet was suspended in 400 μl of fresh TYM before being transferred into a 4-mm-gap cuvette (BTX, Genetronics, Inc., San Diego, CA) with 40 μg of plasmid DNA. Electroporation was performed at 350 V, 950 microfarads, and 1,000 Ω using an ECM 630 Electro cell manipulator (BTX). Following the pulse, cells were placed on ice for 10 min and transferred into a T25 flask with 50 ml of fresh TYM-serum medium without drug for 24 h, at which time 200 μg ml^{-1} Geneticin (G418) (Invitrogen) was added. Single cells were cloned by the addition of individual parasites in 100 μl TYM-serum plus 25 μg ml^{-1} G418 to wells coated with 1 μg FN, as described above.

Growth kinetics. For growth kinetic studies, 1 × 10^6 cells resuspended in 1 ml of TYM containing 200 μg ml^{-1} G418 were inoculated into a fresh T25 flask containing 50 ml TYM growth medium. Growth was monitored over a 24-h period. The organisms were enumerated every 4 h using a hemocytometer.

RESULTS

MAb ws1 inhibits *T. vaginalis* organism binding to immobilized FN. We previously reported the ability of *T. vaginalis* organisms to bind FN (8, 14–16). As shown in Fig. 1A, the addition of trichomonads to FN-coated wells of microtiter plates gave increasing numbers of bound parasites. The binding kinetics are indicative of specific interactions between organisms and FN. We then screened a library of MAbs for the inhibition of parasite-FN associations. Figure 1B shows that an IgG MAb (called ws1) in the supernatant of a cloned hybridoma was effective at inhibition in a concentration-dependent manner, and a 1:2 dilution blocked parasite binding to FN up to 64%.

MAb ws1 detects a 39-kDa protein identified as being GAPDH. We next performed immunoblotting using *T. vaginalis* whole-cell lysate after SDS-PAGE. Nitrocellulose blots were probed with MAb ws1, and as shown in Fig. 2A (lane 1), a 39-kDa protein was detected. As a control, no protein band

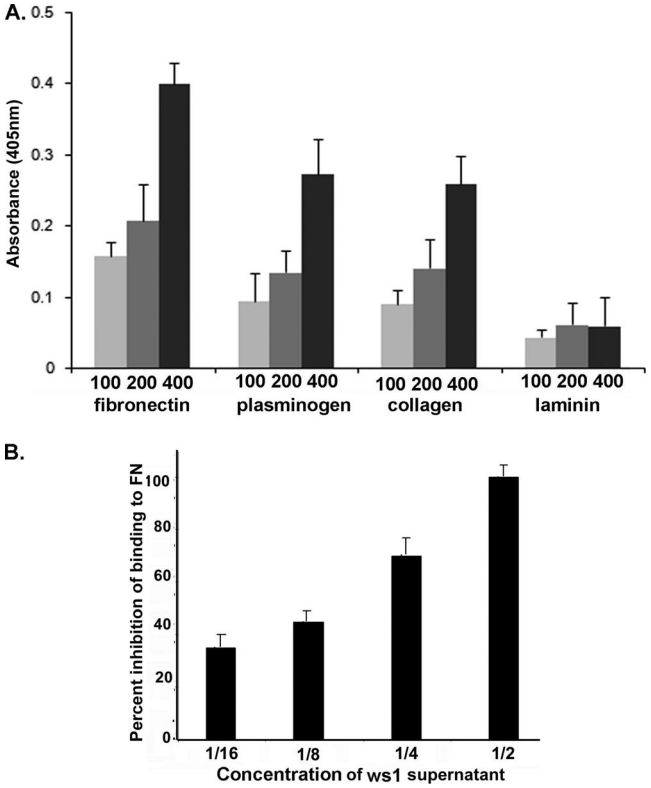

FIG. 3. Representative FN-binding assay with purified rGAPDH and inhibition by MAb ws1. (A) Increasing concentrations of rGAPDH (1,000 to 400 ng/well) were added to microtiter wells coated with 1 μg each of FN, plasminogen, collagen, and laminin. rGAPDH bound to each substrate except laminin in a concentration-dependent fashion. (B) MAb ws1 inhibited binding of rGAPDH to FN. In this assay, 400 ng of rGAPDH was preincubated with different dilutions of MAb prior to the addition of the mixture to immobilized FN on the microtiter wells. MAb ws1 inhibited rGAPDH binding to FN. These results represent the averages of data from four independent experiments with quadruplicate samples.

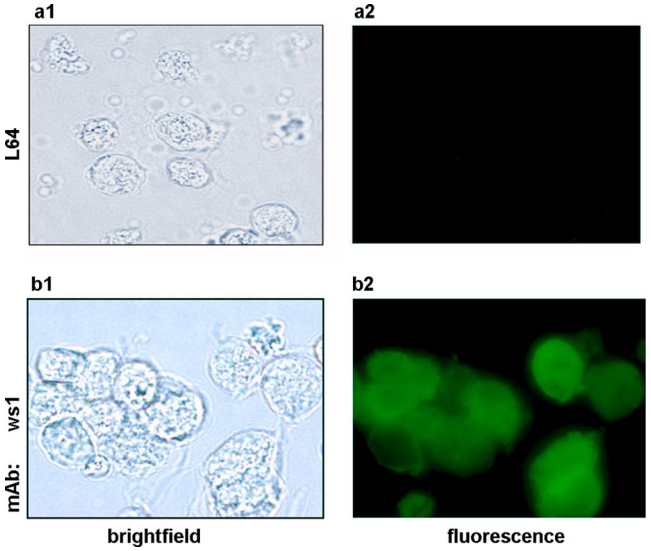

FIG. 4. Fluorescence detection of GAPDH on the surface of nonpermeabilized *T. vaginalis* cells. Paraformaldehyde-fixed, nonpermeabilized cells were incubated with MAb ws1, after which parasites were washed and treated with fluorescein isothiocyanate-conjugated anti-mouse IgG (secondary antibody) (b2). For controls, the *T. vaginalis* cells were treated with MAb L64, which is an antibody to a cytoplasmic protein (27) (a2). Bright-field pictures (a1 and b1) show the integrity of parasites in the same fields of a2 and b2 used for the assay.

was detected in recombinant *E. coli* extracts probed with a MAb of the same isotype but against a different trichomonad protein (data not shown).

We then screened a cDNA library with MAb ws1 as a probe, and two highly reactive clones with overlapping sequences were identified and further characterized. The cDNA sequence of the full-length clone encoded a gene identical to that previously identified as being the trichomonad eubacterium-like GAPDH (32). This full-length cDNA was 1,086 bp with an open reading frame of 362 amino acids and a calculated molecular mass of 39.2 kDa, consistent with the size of the band on the immunoblot (Fig. 2A). Furthermore, the *T. vaginalis gapdh* gene was cloned and the recombinant protein (rGAPDH) was overexpressed in *E. coli* cells (Fig. 2B, lane 2). *E. coli* with vector and without the insert (Fig. 2B, lane 1) was nonreactive with MAb ws1. As expected, the rGAPDH protein was reactive with MAb ws1 (Fig. 2B, lane 3).

rGAPDH binds multiple substrates, and MAb ws1 inhibits binding of rGAPDH to FN. We examined whether GAPDH recognized and bound various substrates. Figure 3A shows the concentration-dependent binding of increasing amounts of rGAPDH added to microtiter wells coated with FN, plasminogen, and collagen. No similar kinetics of binding were evident by rGAPDH on laminin as a control. A reverse binding assay was also performed with the addition of increasing amounts of FN and laminin as a negative control to microtiter wells coated with immobilized GAPDH. Bound FN was detected with anti-FN MAb. The results showed concentration-dependent binding of FN, but not laminin, to GAPDH-coated wells (data not shown).

Next, 400 ng of rGAPDH was pretreated with different dilutions of MAb ws1 in hybridoma supernatant, followed by the addition of the rGAPDH-MAb mixture to immobilized FN. Figure 3B shows that MAb ws1 efficiently inhibited the binding of rGAPDH to FN in a concentration-dependent manner. The 1:2 and 1:4 dilutions inhibited the binding of rGAPDH to FN by 100% and ~70%, respectively, indicating that MAb ws1 is reactive near or at the FN-interacting epitope. Therefore, we wanted to characterize further the localization of GAPDH and its interaction with FN.

GAPDH is on the parasite surface. As shown in a representative experiment in Fig. 4b2, MAb ws1 gave intense fluorescence with the surface of nonpermeabilized trichomonads. No fluorescence was evident with MAb L64, which is reactive with a cytoplasmic protein of *T. vaginalis* (27), as shown in Fig. 4a2. Fig. 4a1 and b1 show bright-field microscopy of the same fields of fluorescence. These results show the presence of the GAPDH on the surface of trichomonads.

Iron regulates expression of GAPDH. There was a marked decrease in amounts of *gapdh* mRNA in *T. vaginalis* parasites grown in medium depleted of iron (Fig. 5A, lane L) compared with organisms grown in iron-replete (lane H) and normal (lane N) medium. Immunoblotting of total proteins of equiv-

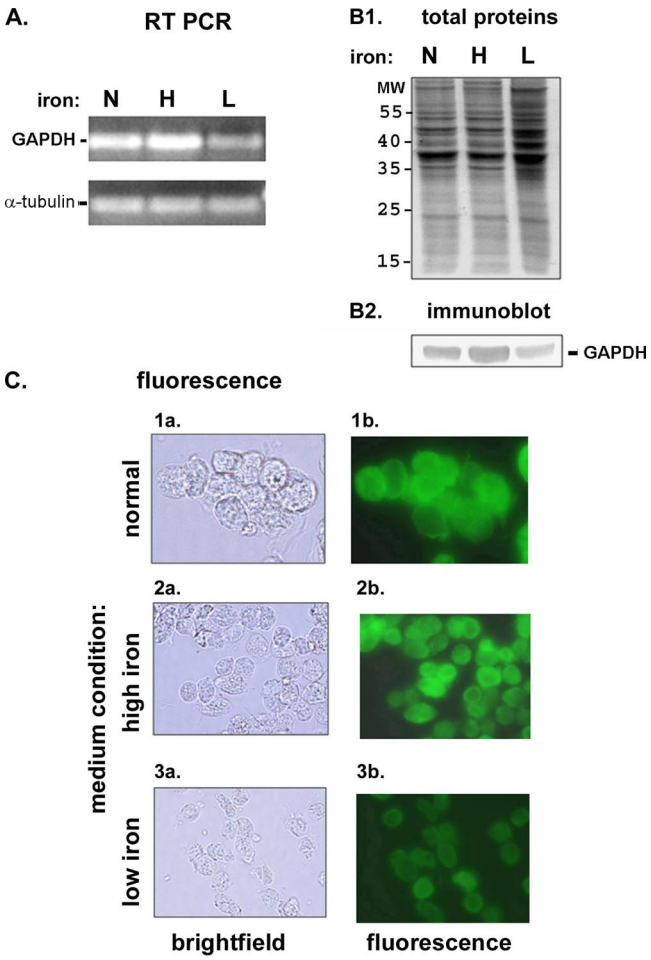

FIG. 5. Expression of GAPDH is regulated by iron. (A) RT-PCR demonstrating the transcription of the *gapdh* gene under normal (N), high-iron (H), and low-iron (L) conditions, as described in Materials and Methods. (Bottom) RT-PCR products for the *T. vaginalis* α-tubulin gene as a control. (B1) Total stained proteins and amounts of GAPDH of equal numbers of trichomonads grown with different concentrations of iron after SDS-PAGE. MW, molecular weight (in thousands). (B2) Duplicate gels were immunoblotted and probed with MAb ws1. (C) Intensities of surface fluorescence of GAPDH in trichomonads grown under different iron conditions. Secondary fluorescein isothiocyanate-conjugated mouse anti-IgG antibody was reacted with nonpermeabilized trichomonads first treated with MAb ws1. The corresponding bright-field pictures show the integrity of the trichomonads used for the assay. As a control, no fluorescence was evident with MAb L64 of the same isotype as MAb ws1.

alent numbers of trichomonads (Fig. 5B1) using MAb ws1 confirmed decreased amounts of GAPDH in the low-iron-grown parasites (Fig. 5B2, lane L) in contrast to high-iron-grown (lane H) and normally grown (lane N) organisms. Furthermore, Fig. 5C shows the decreased intensity of fluorescence of nonpermeabilized trichomonads grown in low-iron medium reacted with MAb ws1 (Fig. 5C3b) compared with organisms grown in high-iron (Fig. 5C2b) or normal (Fig. 5C1b) medium. We also measured the extents of parasite growth under different iron conditions with associations with immobilized FN. Not unexpectedly, higher levels of binding were obtained with high-iron-grown than with low-iron-grown organisms (data not shown). These data reinforce the notion that iron regulates the synthesis and surface placement of GAPDH.

AS inhibition of GAPDH synthesis. Given the multigene family nature of *gapdh* in *T. vaginalis*, we used AS RNA to inhibit the synthesis of GAPDH, as described previously for adhesins (36, 37). We hypothesized that decreased amounts of *gapdh* transcript would correspond to smaller amounts of both surface GAPDH and parasites bound to immobilized FN. PCR was performed to amplify a 795-bp coding region of *neo* using template DNA from wild-type (wt)-, S-, and AS-transfected parasites. Figure 6A shows the *neo* PCR products in S- and AS-transfected trichomonads harboring the plasmids but not in wt control trichomonads. Figure 6C shows the PCR product derived from the plasmid alone as a control.

RT-PCR on total RNA demonstrates decreased amounts of *gapdh* mRNA in AS-transfected parasites (Fig. 6B) in contrast with amounts in S-transfected and wt parasites. Immunoblots of total proteins from numbers of parasites equivalent to those shown in Fig. 5 as described above were performed with MAb ws1 against GAPDH in addition to MAb 12G4 against AP65 adhesin as a control (37). A decreased band intensity detected by MAb ws1 against GAPDH was evident only in the AS-transfected parasites (Fig. 6C). Similar amounts of AP65 were seen in the AS and S transfectants and wt organisms, showing the specific inhibition of *gapdh*. Figure 6D and E show ~80% decreased amounts of transcript and protein, respectively, as evidenced by densitometric scanning of the bands using the Scion Image β program. Furthermore, in Fig. 7A, we show a diminished intensity of fluorescence obtained with MAb on nonpermeabilized, AS transfectants (Fig. 7A3b) versus S transfectants (Fig. 7A2b) and wt parasites (Fig. 7A1b). Last, decreased amounts of GAPDH in AS transfectants did not affect the growth and multiplication of *T. vaginalis*, as evidenced by the fact that no differences in growth kinetics and generation times were detected among the transfectants (Fig. 7B).

Finally, we measured the levels of binding to FN-coated wells by AS trichomonads compared to S and wt organisms. Importantly, as shown in Fig. 8, only the AS transfectants with decreased total amounts of GAPDH (Fig. 6C) and surface-localized GAPDH (Fig. 7) had lower levels of association with immobilized FN than did S transfectants and wt parasites. The extent of FN associations by AS transfectants may be the result of residual GAPDH on the surface. The levels of binding of S transfectants were 18% higher than those of the wt parasites, which is indicative of the increased amounts of GAPDH in these organisms. In separate control experiments performed simultaneously, trichomonads transfected with plasmid without an insert were equivalent to wt trichomonads with regard amounts of GAPDH and levels of binding to FN on microtiter wells. These data suggest strongly that the FN binding is due to surface GAPDH.

Adherence of trichomonads to VECs is unaffected by GAPDH and MAb ws1. We tested whether the trichomonad GAPDH bound to immortalized VECs used by us in adherence assays. No fluorescence was evident using MAb ws1 as a probe on nonpermeabilized VECs first incubated with purified rGAPDH. We then performed inhibition-of-adherence experiments by mixing trichomonads with 1 μg of rGAPDH or by resuspending trichomonads in hybridoma supernatant diluted

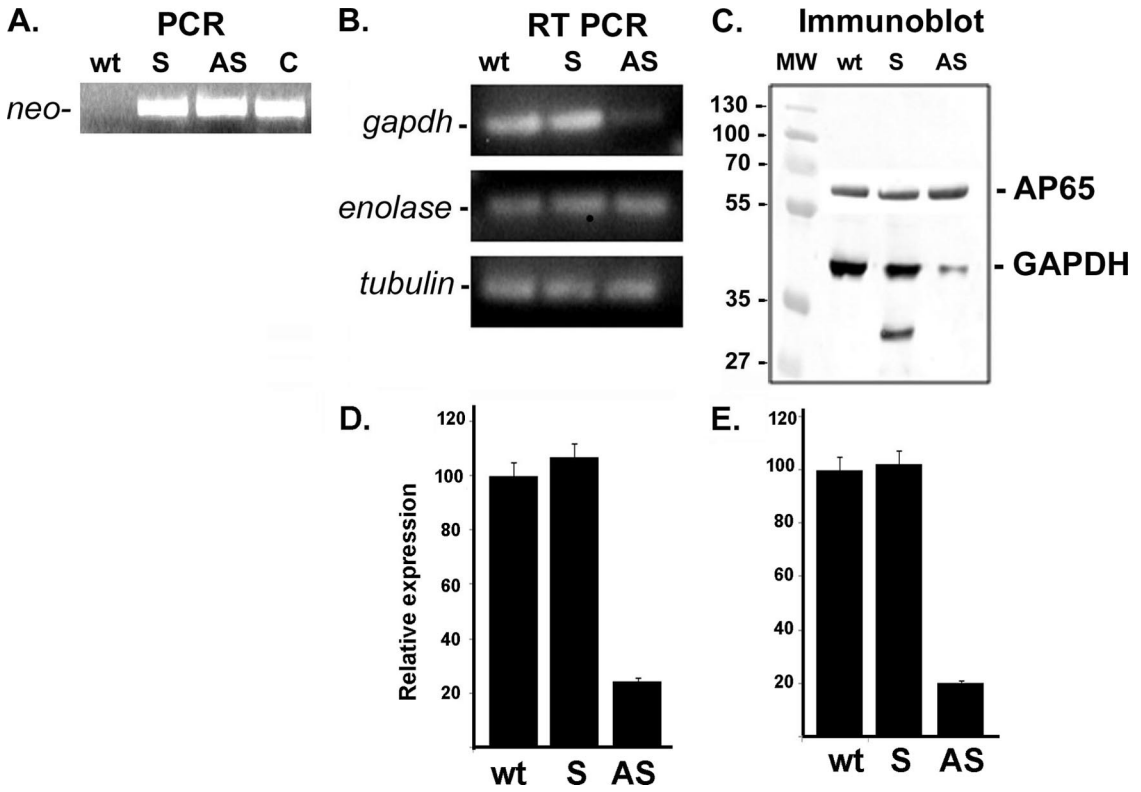

FIG. 6. AS inhibition of synthesis of *gapdh*. (A) PCR amplification of the *neo* coding region in transfected parasites. The ethidium bromide-stained band after electrophoresis in 1% agarose is the PCR product of the *neo* gene that was amplified using DNA from transfected *T. vaginalis* parasites. As expected, no PCR band was obtained from wt organisms (lane wt), and a predicted product was obtained from the plasmid used directly during PCR as a control (lane C) and the S- and AS-transfected parasites. (B) Representative experiment showing confirmation of *gapdh* gene expression patterns of wt and S- and AS-transfected trichomonads by semiquantitative RT-PCR. One microgram of total RNA was reverse transcribed using primer oligo$(dT)_{12}$, and PCR was performed using gene-specific primers. The α-tubulin and *eno1* genes served as controls. (C) Immunoblot analysis of *T. vaginalis* cell lysate showing the decrease in amounts of GAPDH in AS trichomonads compared to amounts in wt and S-transfected trichomonads. As a control to show equivalent amounts of protein in each lane, the same blot was probed with antibody 12G4 against AP65 (19, 22, 37). MW, molecular weight (in thousands). (D and E) Bar graphs showing the relative amounts of the RT-PCR products for the *gapdh* transcript and amounts of protein, respectively. The amount of wt *gapdh* transcript was normalized to 100%. Quantitation was done by densitometric scanning of the bands and by using the Scion Image β program.

1:2, which gave an efficient inhibition of binding of parasites to immobilized FN (Fig. 3). As shown in Fig. 9, compared with untreated trichomonads handled identically, there was no detectable inhibition of adherence by either GAPDH or MAb. Importantly, we included another sample within the same experiment to show adherence mediated by the AP65 adhesin (10, 20, 37). As expected, the pretreatment of organisms with rabbit anti-AP65 serum gave a decrease in the level of adherence to VECs (Fig. 9) compared to untreated, control trichomonads treated with normal rabbit serum that gave results equal to those shown in Fig. 9.

DISCUSSION

We isolated and identified a surface-associated GAPDH from *T. vaginalis* that was found to bind to FN, plasminogen, and collagen but not laminin. A MAb directed against the parasite surface with the property of inhibiting parasite associations with immobilized FN detected a 39-kDa protein identical to the trichomonad eubacterium-like GAPDH and possessed an insertion in positions 115 to 126 that is unique to *T. vaginalis* (32). The significance of this insertion with respect to enzymatic activity or other alternative functions is unknown. This stretch of amino acids contains many polar and charged residues, some that may be involved in protein-protein interactions. Because of the efficient inhibition of parasite associations with FN, we characterized further the GAPDH-FN interaction. Of interest is the lack of an FN-binding consensus sequence (23) within the trichomonad GAPDH, suggesting a different as-yet-unknown mechanism of GAPDH binding to FN. The finding that the MAb inhibits both organisms and purified GAPDH (and rGAPDH) interacting with FN suggests that the MAb is directed toward a site at or near the FN-binding epitope.

GAPDH is a cytoplasmic enzyme involved in the glycolytic pathway responsible for the phosphorylation of glyceraldehyde-3-phosphate to generate 1,3-biphosphoglycerate. The identification of numerous diverse biological properties of mammalian GAPDH proteins is now well established (42). These include roles in transport and membrane fusion, microtubule assembly, nuclear RNA export, and protein phosphotransferase/kinase reactions. The presence of the GAPDH protein on the bacterial surfaces of *Streptococcus pyogenes*,

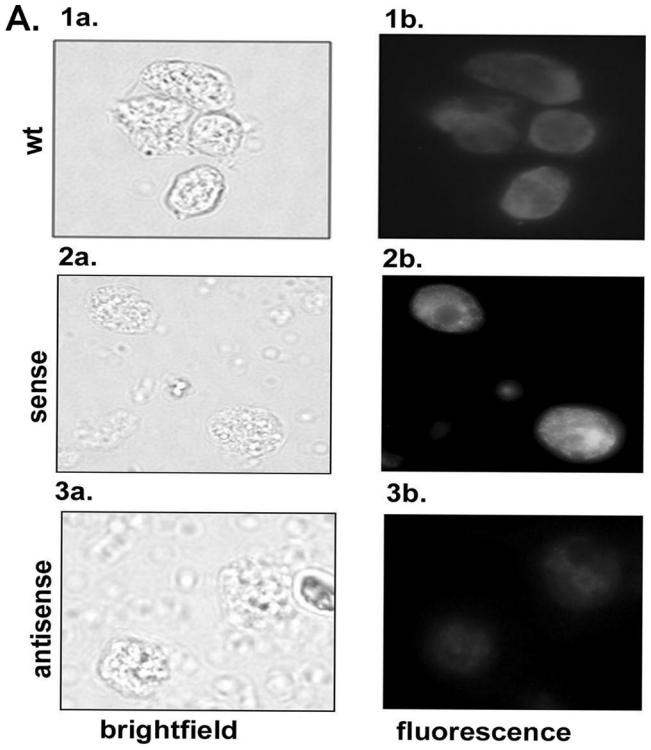

FIG. 7. AS transfectants show decreased surface fluorescence and no effect on trichomonal growth patterns. (A) Immunofluorescence of nonpermeabilized parasites shown in Fig. 4 shows decreased levels of surface GAPDH (3b) on AS versus S transfectants (2b) and wt trichomonads (1b). (B) Growth profile of wt and S- and AS-transfected trichomonads. Parasites were enumerated at different time points using a Neubauer hemocytometer. Similar results were obtained for three independent growth experiments performed on different days.

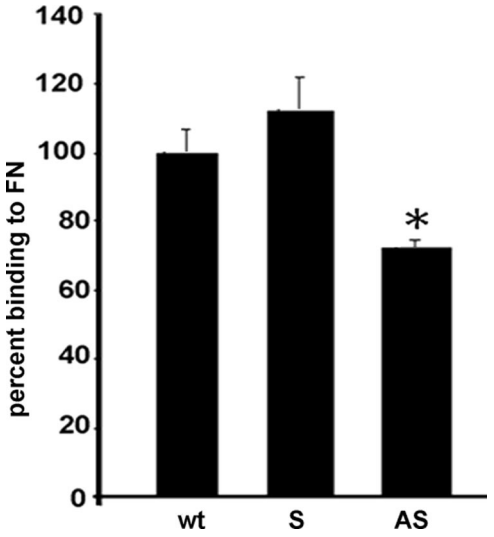

FIG. 8. Relative *T. vaginalis* binding to FN by AS trichomonads compared to those by wt and S trichomonads. The extent of binding by wt organisms was normalized to 100% for comparative purposes. The results are the averages of data from three different experiments performed at different times with quadruplicate samples, and the decrease in the level of associations of AS transfectants with FN was statistically significant (asterisk) ($P < 0.05$).

Streptococcus pneumoniae, and, more recently, *Streptococcus agalactiae* was previously reported (11, 30, 34, 38). This enzyme has multiple functional activities and contributes to the virulence of pathogenic streptococci by means of binding to host proteins including plasminogen/plasmin, actin, and FN (11, 17, 22, 34, 38). Thus, it seems that GAPDH on the microbial surface has implications for virulence and pathogenesis due to its functional diversity.

Immunofluorescence studies demonstrated the enzyme on the surface of trichomonads (Fig. 4). Recent studies have shown that *T. vaginalis* AP65/decarboxylating malic enzyme, GAPDH, α-enolase, and other metabolic enzymes are secreted or released into the medium (27). Furthermore, AP65 and α-enolase were found to be associated with the parasite surface, indicating that they are members of a family of anchorless, surface-associate enzymes with alternative functions, as described previously for other bacteria, fungi, and parasites (17, 19, 35, 38, 39). Thus, our data support the notion that GAPDH is also a member of the family of surface-associated enzymes, and in this case, GAPDH is a receptor for FN, among other substrates.

In this study, we show that iron influences the expression of GAPDH at the transcriptional level and, therefore, affects the synthesis and surface placement of the protein (Fig. 5). Furthermore, the data indicate that iron coordinately regulates the expression of the *gapdh* multigene family. If this were not the case, we would expect that amounts of total mRNA and GAPDH protein would not decrease due to gene family members that are unresponsive to iron. While growth of trichomonads in iron-depleted medium reduces the virulence of *T. vaginalis* (41), we know that most of the trichomonads in vivo are of the high-iron phenotype (7), indicating the constitutive expression of GAPDH during infection. Of particular interest is that the pretreatment of low-iron-grown trichomonads with MAb ws1 did not further lower the extent of parasite binding to FN, and this indicates that there may be other FN-binding ligands that are yet to be identified.

Although speculative, it is reasonable to consider a role for GAPDH in providing the parasite with a survival advantage. For example, barrier disruption of the vaginal epithelium may lead to persistence (40) through the anchoring of trichomonads with basement membranes, as hypothesized previously (14). This expression of FN receptors on the parasite

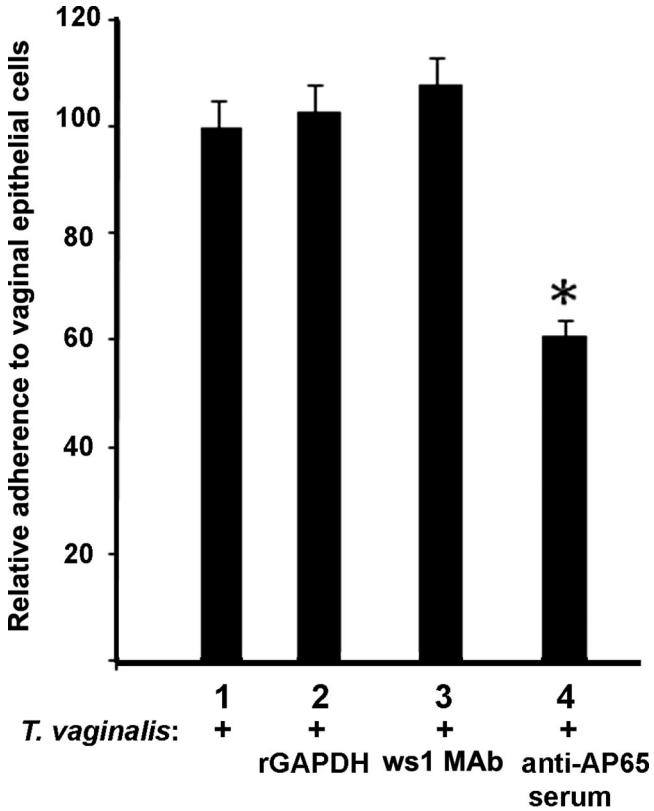

FIG. 9. Adherence of *T. vaginalis* organisms to MS74 VECs. Washed logarithmic-phase trichomonads were untreated, treated with 1 μg of rGAPDH, or suspended after washing with hybridoma supernatant diluted 1:2 containing MAb ws1 prior to adding the mixture to cell monolayers. Another sample included trichomonads suspended in antiserum against AP65 (19, 21, 37) diluted 1:2 in medium prior to addition to host cells.

would permit access to distinct host sites with unique nutritional and physicochemical environments that may be a prerequisite for colonization (15) and, perhaps, persistence (40). This places GAPDH in the same category of importance as is recognized for the expression of adhesins for adherence to VECs (36, 37).

Our work with AS RNA inhibition of synthesis of the surface adhesins AP65 and AP33 provided additional confirmatory evidence for a role of these proteins in *T. vaginalis* adherence to VECs (36, 37). Given the multigene family nature of trichomonad GAPDH, we used AS RNA inhibition of the synthesis of this enzyme to show decreased amounts of surface-associated protein. Not unexpectedly, this yielded lower levels of FN binding by live, AS-transfected trichomonads. The absence of a complete inhibition of binding is not likely given the lack of total inhibition of synthesis of the protein and the presence of residual GAPDH on the surface, as was also the case for low-iron-grown trichomonads.

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